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Effects of Bretylium Tosylate on Voltage-Gated Potassium Channels in Human T Lymphocytes

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SUMMARY

Using the patch-clamp technique, we determined that bretylium tosylate, a quaternary ammonium compound possessing immunomodulating activity, decreased the whole-cell K^+ current in human T lymphocytes, in a dose-dependent manner, in the 0.05–5 mM extracellular concentration range. Bretylium tosylate prolonged the recovery from inactivation and accelerated the inactivation and deactivation of the K^+ current but did not influence the kinetics of activation or the voltage dependence of activation and steady state inactivation of the K^+ conductance. The percentage of drug-induced block was independent of membrane potential. K^+ channel block by bretylium tosylate was partially and slowly removable by washing with drug-free extracellular solution. Bovine serum albumin (10 mg/ml) in the bath lifted the drug-induced block almost instantaneously, although not completely. In control experiments bovine serum albumin increased the inactivation time constant of the K^+ channels but left the peak K^+ current amplitude unaffected. On the basis of the experimental evidence, a gating-dependent allosteric interaction is suggested for the mechanism of drug action. The effective dose range, time of exposure, and reversibility of bretylium tosylate-induced K^+ channel block correlated well with the same parameters of the drug-induced inhibition of T lymphocyte activation. The reported effects of bretylium tosylate on T cell mitogenesis can be regarded partly as a consequence of its blocking effects on voltage-gated K^+ channels.

BT, a drug known for its antifibrillatory and antihypertensive potency, effectively increases the activity of the amiloride-sensitive sodium channels of epithelium and also influences Na^+ and K^+ currents in heart cells (1–3). Recently, it has been shown that BT influences alternate cell activation processes (one involving the phosphatidylinositol bisphosphate cascade, activating protein kinase C, and the other working via interleukin-2 signaling) in human lymphocytes via its influence on the resting potential of the cells (4–8). It has been shown, by measuring [^{3}H]dThd incorporation into the cells, that the inhibitory effect of BT on the proliferative response of PHA-treated human peripheral T lymphocytes is dose dependent in the 0.25–2.0 mM range, with a half-maximal inhibitory concentration of approximately 1 mM. The inhibitory effect of BT lasted for as long as the cells were incubated with the drug; however, removal of BT by washing of the cells with 10% fetal calf serum-containing medium lifted the inhibition of proliferation immediately (8). In connection with this effect, a BT-induced transient sodium current has been identified in human lymphocytes (9). It is well known that ion channel activity, especially that of voltage-gated K^+ channels, and membrane potential changes may influence mitogen-induced cell activation processes (10–12). We were therefore interested in the possible effect of BT on the K^+ channel activity of human T lymphocytes.

Four lines of evidence suggest a role for K^+ channels in the proliferation of T cells. First, mitogen-induced hyperpolarization is accompanied by an increase in the K^+ permeability of the cell membrane (12). Second, mitogen-activated T cells undergo (during hours/days) a moderate increase in whole-cell conductance due to an increased number of voltage-gated K^+ channels in the membrane, although the short term (minutes/hours) effects of mitogen stimuli on the whole-cell voltage-gated K^+ conductance of human T cells are controversial (13). Third, a variety of K^+ channel blockers, including quinine, verapamil, nifedipine, diltiazem, tetraethylammonium, charybdotoxin, noxiustoxin, and margatoxin, inhibit K^+ currents

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ABBREVIATIONS: BT, bretylium tosylate (*N*-ethyl-*N*-*o*-bromobenzyl-*N*,*N*-dimethylammonium tosylate); dThd, thymidine; PHA, phytohemagglutinin; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

and mitogen-stimulated proliferation with similar rank orders of potency (14–17). Fourth, the C3H-*lpr/lpr* mouse strain develops a lupus-like syndrome accompanied by hyperplasia of a functionally and phenotypically abnormal T cell subset. These aberrant cells display an abundance of L-type K^+ channels (18).

The involvement of voltage-gated K^+ channels in lymphocyte activation is relatively well understood. The membrane potential of lymphocytes is primarily determined by the K^+ diffusion potential (13, 19, 20); therefore, it is most reasonable that K^+ channels maintain a "permissive" membrane potential for the initiation and propagation of the mitogenic signal. K^+ channel blockers depolarize T cells (16) and depolarization inhibits Ca^{2+} signaling (17), thereby supporting the idea that K^+ channels indirectly facilitate Ca^{2+} influx through mitogen-stimulated Ca^{2+} channels by maintaining a permissive negative membrane potential.

In the present study the effect of extracellular BT on the voltage-gated K^+ channel activity in human peripheral blood T lymphocytes has been studied by the patch-clamp method. We show that macroscopic properties of voltage-gated K^+ channels in the plasma membrane of T lymphocytes are influenced by the drug.

Materials and Methods

BT was purchased from Serva (Heidelberg, Germany), and all other chemicals were from Sigma Chemical Co. (St. Louis, MO). Peripheral blood was obtained from healthy volunteers, and T cells were purified from peripheral blood lymphocytes as described elsewhere (21). Ion currents were recorded with Axopatch-200 and -200A patch-clamp amplifiers in conjunction with Axon Instruments TL-1-125 computer interfaces with varying sampling rates, applying low-pass filtering at half the sampling frequency. All patch-clamp experiments were carried out at room temperature. Patch electrodes, of 3–4-MΩ resistance, were fabricated from GC 150 F-15 glass capillaries (Clark Electromedical Instruments, Reading, England). The pipettes were filled with a KF pipette solution (140 mM KF, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, pH 7.2). The cells were bathed in Ringer solution (160 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.4), unless otherwise stated. The measured osmolarity of the solutions was 300–340 mOsM. Gigaseals on the order of 20–100 GΩ were formed and whole-cell measuring configurations were then established in the usual manner. Control experiments were usually started at least 12 min after establishment of the whole-cell configuration, and with the KF internal solution K^+ channel properties of T lymphocytes remained stable for up to several hours (22). Expected R_s (series resistance) values were ≤12 MΩ ($3 \times R_{pip}$; R_{pip} = pipette resistance), whereas maximal G_K (potassium conductance) was on the order of 1 nS, which implies measured G_K errors of ≤12%. During the experiments no series resistance compensation was used. After the initial setting, there was no need to alter the capacitive transient compensation, which means that after the initial equilibration period no tendentious changes in R_s occurred during the long term experiments or after solution changes. Regarding time control comparisons, peak K^+ current amplitudes at a given depolarizing potential usually remained within ±5% of the control value for several hours, after which abrupt loss of the cell occurred. Cells displaying larger deviations during control experiments after the initial equilibration were discarded. In most of the experiments leak current was negligible, compared with the K^+ current; therefore, during the analysis of the results no leak subtraction was done, except when specifically stated. Results are usually given as mean ± standard error for a given number of cells, *n*. Whenever error bars are not displayed they fall within the limits of the symbols used.

Results

Fig. 1a illustrates that, under typical voltage-clamp conditions, human T lymphocytes display strong outward whole-cell currents upon step depolarization and the current is activated more rapidly with increasing depolarization. After reaching a peak, the current declines, due to inactivation, toward a low steady state level. Ion channels contributing to this transient outward current have been identified as voltage-gated potas-

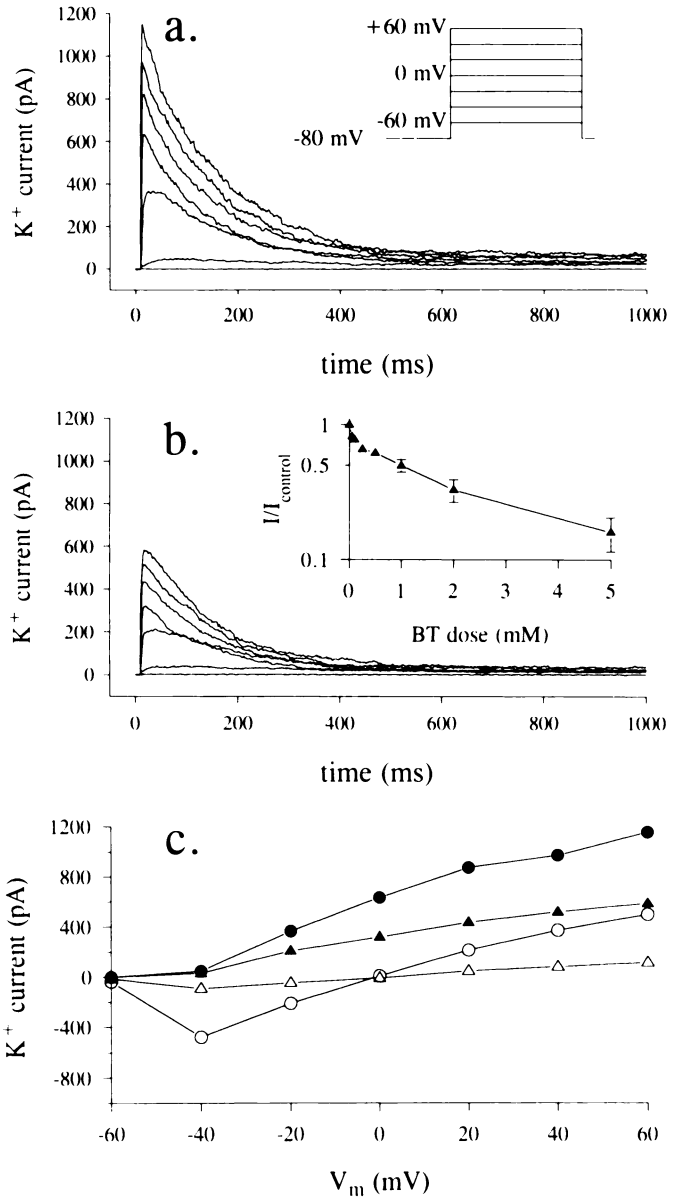

Fig. 1. Partial block of whole-cell K^+ current by externally applied BT in human T lymphocytes. a, Control. *Inset*, voltage steps lasting for 1000 msec were applied every 150 sec from a holding potential of −80 mV in 20-mV increments from −60 to +60 mV. b, After 7 min of incubation with 1.0 mM BT in the bath. *Inset*, cumulative dose dependence of partial potassium channel block by BT in human T lymphocytes (n = 4) is shown. The $I/I_{control}$ values are based on peak current values at +20-mV test potential before and after the application of BT. c, Effect of extracellular BT on peak current-voltage relations of whole-cell K^+ currents in a T lymphocyte in the Ringer solution (●, control; ▲, 1.0 mM BT) and under K^+-depolarized (external potassium concentration, 165 mM) conditions (○, control; △, 1.0 mM BT). No leak current subtraction has been applied.

sium channels on the basis their being selective for K^+, having a linear single-channel current-voltage relationship with a slope conductance of approximately 12 pS at pipette potentials between −30 and +40 mV, and properly responding to quinine (100 μM) and 4-aminopyridine (5 mM) (19, 22).

The prolonged (5–10-min) presence of 1.0 mM BT in the external medium significantly reduced peak K^+ currents in T lymphocytes (Fig. 1b). The BT-induced potassium channel block was dose dependent in the 0.05–5 mM concentration range ($n = 4$), when measured with cumulative doses (Fig. 1b, *inset*). Cumulative dose dependence was determined because recovery upon BT removal was only partial (see below). The degree of inhibition by 1.0 mM BT was similar (50 ± 5.3%, $n = 5$) over the entire voltage range studied ($V_m \geq -30$ mV), i.e., inhibition was voltage independent (Fig. 1c). This, in itself, is good evidence that the BT effect was not due to an increase in series resistance, because in that case only the larger conductances would have been influenced.

A comparison of the current-voltage relationships in K^+-depolarized human T lymphocytes before and after the application of BT showed no preferential blocking of the flow of potassium ions in either direction. Both inward and outward currents were decreased to approximately 20% of their original values by 5 mM BT (Fig. 1c).

Fig. 2 displays the effect of BT on the voltage-dependent activation and steady state inactivation profiles of the transient K^+ current. The voltage dependence of normalized peak chord conductance, determined at different test potentials (Fig. 2a), and normalized peak K^+ currents, measured at a constant test potential (+20 mV) after inactivation at different holding potentials (Fig. 2b), were both unaffected by the drug, excluding the possibility of a charge-screening mechanism during the action of BT.

The effect of externally applied BT on the kinetics of the potassium current in T lymphocytes was then studied in more detail. Marked effects on the kinetics of deactivation of potassium channels in T lymphocytes were observed. Fig. 3 illustrates the results of a typical tail-current experiment in the absence and presence of 2.5 mM BT. Inspection of the tail currents revealed that BT increased the rate of deactivation in T lymphocytes by approximately 75% throughout almost the entire membrane potential range studied (Fig. 3b, *inset*). The effect of BT on the driving force of potassium ions could also be deduced from the tail currents. The tail currents reversed at approximately −70 mV in the control experiments and a small but significant shift of the reversal potential in the positive direction (+5.3 ± 4.2 mV, $n = 9$, $p = 0.01$) was observed, suggesting a slight change in the ion selectivity of the K^+ channels due to BT, in accord with earlier observations (9).

Inactivation of the K^+ conductance was studied using a voltage protocol similar to that of Fig. 1 ($n = 6$). BT slightly (approximately 20%) but significantly increased the inactivation rate in the membrane potential range of −20 mV to +60 mV ($n = 6$). We demonstrated the effect of BT at a fixed test potential because the inactivation rate is voltage dependent. At 0-mV test potential 1.0 mM BT altered the time from peak current to half-inactivation from 153.4 ± 13.6 msec to 108.7 ± 10.6 msec ($n = 6$, $p = 0.001$).

BT considerably increased the time needed for the potassium channels to recover from inactivation when the K^+ currents of T lymphocytes were inactivated by clamping the membrane

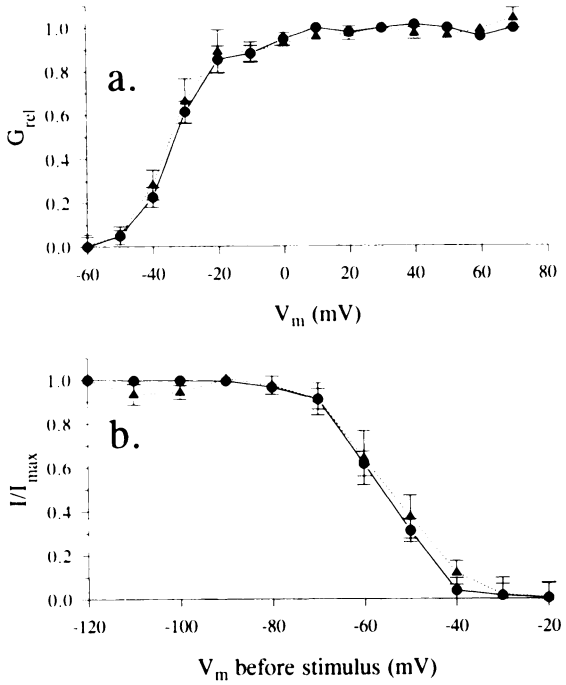

Fig. 2. Effect of extracellular BT on the voltage dependence of activation (a) and steady state inactivation (b) of K^+ channels. a, Normalized peak chord conductance-voltage relationships (G_{rel} versus V_m; $G_{rel} = G_K/G_{K_{max}}$, where $G_{K_{max}}$ is the maximal value of G_K) for whole-cell K^+ current in human T lymphocytes ($n = 6$) without (●) and with (▲) externally applied 1 mM BT, after leak subtraction. The whole-cell K^+ currents originate from voltage-clamp experiments similar to those of Fig. 1. Leak current was estimated by linear extrapolation on the basis of currents recorded at subthreshold potentials. b, Voltage dependence of steady state inactivation of K^+ current in human T lymphocytes without (●) and with (▲) externally applied 1 mM BT. Currents were elicited by a constant test potential to +20 mV after 3 min of equilibration of K^+ channel inactivation at the displayed holding potentials. Peak currents at different holding potentials were normalized to that measured at −120 mV and were averaged for four independent experiments before and after the addition of BT.

potential of the cells at a depolarizing potential, e.g., −20 mV (Fig. 4a). Inactivation of the potassium channels developed during maintained depolarization and a new level of channel activity was established after a change in the holding potential to −80 mV. Assuming a first-order process, time constants characterizing recovery from inactivation could be deduced from the experiment (control, $\tau = 17.2 \pm 3.6$ sec; 2.5 mM BT, $\tau = 31.4 \pm 2.0$ sec; $n = 3$) (22). The alteration by BT of the time course of recovery from inactivation was further supported by many experiments ($n \geq 50$) in which the effect of BT on other properties of the voltage-gated K^+ conductance was investigated; after the administration of BT longer interpulse intervals ($\Delta t \geq 120$ sec) had to be selected, compared with the control values, using a two-pulse experiment and varying the time interval between the pairs of identical pulses (80 msec, to +20 mV).

In contrast to the alterations in the deactivation and inactivation of the potassium conductance described above, no significant effect of BT on the activation kinetics of K^+ channels in T lymphocytes was observed (time to half-activation at +20-mV test potential: control, 4.96 ± 0.74 msec; 1.0 mM BT, 5.44 ± 0.77 msec; $n = 9$).

The potassium channel block caused by BT was partially and very slowly removable by replacement of the BT-contain-

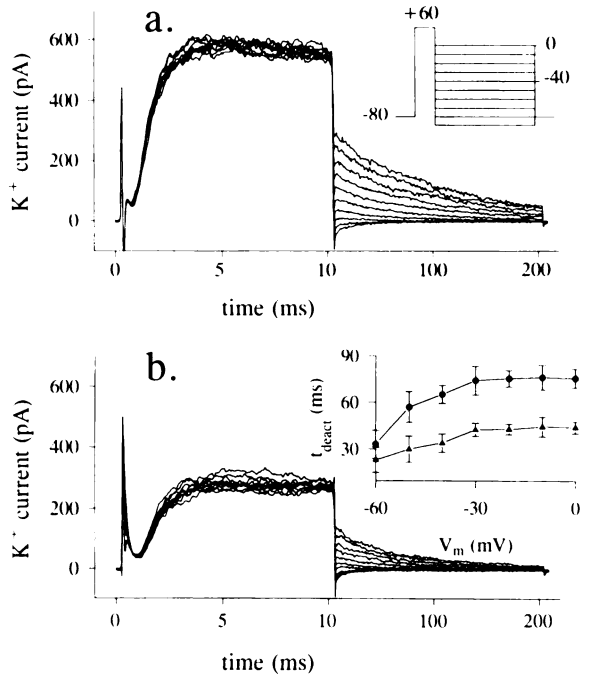

Fig. 3. Effect of extracellular BT on the voltage dependence of the deactivation of the K^+ current in human T lymphocytes. a, Control. *Inset*, the current was activated by 10-msec (duration) voltage steps to +60 mV at regular intervals of 120 sec, from a holding potential of −80 mV. Deactivation was initiated by steps back to more negative potentials. b, With 2.5 mM BT. *Inset*, deactivation became faster due to the application of the drug to the bath (●, control; ▲, 2.5 mM BT; $n = 9$). Decativation time constants were determined by fitting single-exponential functions to the tail currents.

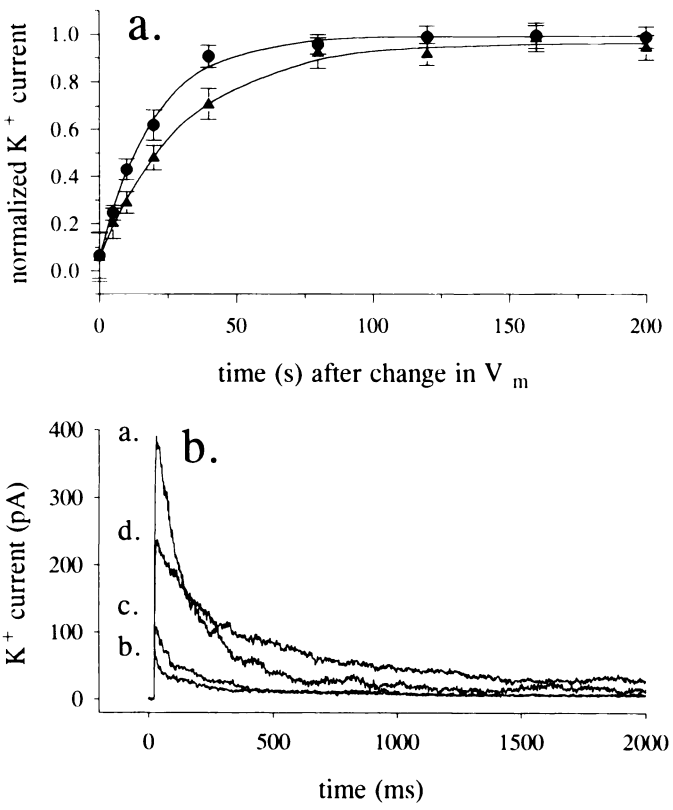

Fig. 4. a, Effect of extracellular BT on the time course of recovery from K^+ channel inactivation in human T lymphocytes after the holding potential was changed from −20 mV to −80 mV. *Points* show peak currents during properly timed 40-msec pulses to +40 mV. The first point at time 0 shows the test current at the previous holding potential (−20 mV). Normalized currents from three independent experiments were averaged before (●) and after (▲) the addition of 2.5 mM BT. Assuming a simple exponential recovery [$I(t) = I_o \exp(-t/\tau) + I_{stat}$], the following parameters were determined by least-squares fitting of the formula to the experimental data: control, $I_o = -0.9145$, $I_{stat} = 0.9786$, $\tau = 17.2$ sec; BT, $I_o = -0.9093$, $I_{stat} = 0.9650$, $\tau = 31.4$ sec. b, Partial removal of the extracellular BT-induced K^+ channel block from a human T lymphocyte by washing. *Trace a*, control; *trace b*, 5 min after the addition of 5 mM BT to the bath; *trace c*, 1 hr after exchange of the external medium for BT-free Ringer solution; *trace d*, 1 min after the application of 10 mg/ml BSA to the bath.

ing external medium with the Ringer solution used in the control measurements ($n = 5$). However, when the BT-containing external medium was exchanged for 10 mg/ml BSA (dissolved in normal Ringer solution), an almost instantaneous but incomplete removal of the blocking effect of BT was observed (from 15.9 ± 2.1% to 60.7 ± 4.0%, $n = 6$), in conjunction with a slower inactivation of the K^+ channels characterized by a time to half-inactivation of 174.7 ± 10.7 msec ($n = 6$), which is a slightly higher value than that for the control measurements (Fig. 4b). Control experiments ($n = 5$) in the absence of BT proved that BSA had no effect on the peak K^+ current amplitude but did alter the inactivation time constant of the K^+ channels in a way similar to that described above for the partial recovery from BT block.

Discussion

Antagonists of the K_n channels (n-type K channels) in T lymphocytes are numerous and have various effects on the kinetics and voltage dependence of channel activity. Detailed mechanisms of channel block have been proposed in only a few cases (19, 20). In recent years two basic mechanisms of channel block have emerged. Both of them should be considered for BT as a blocking agent. One suggests direct entry of the drug into the pore, from either the external or the internal end. In this case block of open channels may be voltage dependent and reversed by ions from the opposite side. This mechanism is contradicted by our observations, because BT had no influence on the voltage dependence of the activation and steady state inactivation of the K^+ conductance and caused no preferential blocking of ion movement in either direction. The other mechanism proposes that the action of the drug takes place at a regulatory or allosteric site on the intra- or extracellular surface, stabilizing closed conformational states of the pore so that opening becomes less likely and the inactivation rate may increase (20). BT decreased the whole-cell K^+ currents and significantly accelerated their inactivation without influencing the voltage dependence of activation and steady state inactivation; therefore, we find this second mechanism of block more likely for BT.

We showed that the initial rise of the K^+ current was not affected by BT, probably because the channel block by BT developed during a lag period (5–10 min), when the K^+ channels spent part of the time spontaneously in the open state at negative holding potentials (equal to or more negative than −50 mV). Even if the drug does not bind in the pore, conformational changes of the channel protein linked to the openings may facilitate the access of the drug to a separate site of action. For the slowly developing K^+ channel block another plausible explanation would be the internalization of BT and its subse-

quent action via a separate intracellular binding site. This reasoning is contradicted by the fact that BSA removed the blocking effect of externally applied BT almost instantaneously. We did expect this effect of BSA on the recovery of K^+ conductance from external BT block, on the basis of the drug-binding abilities of albumins and the BT-binding ability of plasma proteins, as well as our earlier [^{3}H]dThd incorporation experiments (8, 23, 24). However, the effect of BSA on the inactivation rate was a surprising result. Because circulating T lymphocytes *in vivo* are continuously exposed to serum albumin, the observation implies that K^+ conductance inactivation *in vivo* may be slower than in the *in vitro* experiments. The mechanism of this BSA-induced slowing of inactivation is still unclear (direct interaction with the channel protein, effects on surface charges, etc.,) but may be of importance for inactivating K^+ conductances in other blood cells, such as B cells and macrophages (19, 20). The fast recovery from BT block in the presence of BSA is very likely due to direct binding of BT to BSA, causing the removal of the drug from the channel protein.

In summary, this is the first report to examine the effects of BT on the voltage-dependent, whole-cell, K^+ currents of human T lymphocytes. We showed that voltage-gated potassium channels of human T lymphocytes were sensitive to externally applied BT, with a half-blocking concentration of 1 mM, a concentration producing 50% inhibition of [^{3}H]dThd incorporation into PHA-treated human peripheral T lymphocytes. Both K^+ channel block and PHA-induced [^{3}H]dThd incorporation inhibition depended on the presence of BT in the extracellular medium, and both effects could be removed almost instantaneously by washing the cells with solutions containing serum albumins (8). Because of its characteristics demonstrated above, BT may be regarded as a member of the family of K^+ channel blockers known to influence T cell proliferation (25, 26).

Acknowledgments

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